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Anal Biochem. Author manuscript; available in PMC 2018 December 04.

Published in final edited form as:

Anal Biochem. 2017 November 01; 536: 8–15. doi:10.1016/j.ab.2017.08.003.

Methods for Extracting and Characterizing RNA from Urine: for downstream PCR and RNAseq Analysis

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Abstract

Readily accessible samples such as urine or blood are seemingly ideal for differentiating and stratifying patients; however, it has proven a daunting task to identify reliable biomarkers in such samples. Noncoding RNA holds great promise as a source of biomarkers distinguishing physiologic wellbeing or illness. Current methods to isolate and characterize RNA molecules in urine are limited. In this proof of concept study, we present a method to extract and identify small noncoding RNAs in urine. Initially, quantitative reverse transcription PCR was applied to confirm the presence of microRNAs in total RNA extracted from urine. Once the presence of micro RNA in urine was confirmed, we developed a method to scale up RNA extraction to provide adequate amounts of RNA for next generation sequence analysis. The method described in this study is applicable to detecting a broad range of small noncoding RNAs in urine; thus, they have wide applicability for health and disease analyses.

Keywords

noncoding RNA; extracellular RNA in urine; ovarian cancer; small RNA next generation sequencing; tRNA fragments

1. Introduction

Physiologically representative and accessible samples such as saliva, blood or urine have long been expected to provide a source of biomarkers with high potential for characterizing conditions of health and disease. These types of samples are referred to as liquid biopsies and may harbor circulating cells, protein, DNA, and RNA biomarkers (1). RNA is one component within these samples that was initially ignored due to its propensity for rapid

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degradation by ribonucleases (RNases). However, with the identification of microRNAs and their notable stability in physiologic samples, RNA has come to the forefront of readily accessible molecules for the discovery of novel biomarkers (2–4).

Noncoding RNAs (ncRNAs) found in liquid biopsies include, but are not limited to, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), tRNA fragments (tRFs) and microRNAs. The wide variety of functions of extracellular ncRNAs are currently under investigation. For example, the tRFs, although having the unfortunate name of tRNA fragments, are actively processed from mature tRNAs and their function is beginning to be elucidated (5). While the microRNAs are already known to function in most if not all biological processes. Circulating microRNAs as serum biomarkers of health and disease have been robustly explored (6–8); however, the microRNA repertoire in urine is less studied. The majority of the early studies of RNA in urine were focused on prostate, bladder and kidney disease because these tissues would directly contribute extracellular vesicles (EVs) to urine (9,10). In many of the studies of EVs in urine, they are referred to as exosomes due to their size of approximately 40–100 nm. The term EV refers to any extracellular vesicle including but not limited to the microvesicles, ectosomes and exosomes (11). The misconception that extracellular RNAs (exRNAs) must be associated with EVs persists despite recent studies that show exRNAs may be associated with protein and lipid complexes independent of vesicles (12–14). These findings suggest that exRNA in urine may provide useful biomarkers of physiological relevance to many diseases not limited to those involving urologic disease (15).

Urine based biomarkers would be ideal for many studies because of the accessible nature of urine. Urine is readily collected in many animal models, as well as in the veterinary setting and for human health assessment. A urine-based RNA biomarker for ovarian cancer would be particularly useful due to the inaccessible nature of the ovaries and the ability of this cancer to metastasize with few symptoms (16–18). Studies of microRNAs in ovarian cancer have delineated a set of differentially expressed microRNAs that are expected to regulate key tumor suppressors such as BRCA1 (19). Such oncomirs would be expected to be over expressed in biological fluids from cancer patients and have been studied in tissue, serum, ascites fluid and urine from ovarian cancer patients (20–24). microRNAs associated with extracellular vesicles (EVs) are also found in urine from healthy volunteers (25,26). It is possible to isolate EVs from large amounts of urine by a combined method of filtration and ultracentrifugation (25,26); however, a rapid method to isolate total RNA from small amounts of urine is needed. In this article, we present a proof of concept study of the isolation of total RNA from urine allowing either quantitative reverse transcription-PCR (qRT-PCR) or next generation sequencing (NGS).

2. Materials and methods

2.1. Participants and Sample Collection

The Gynecologic Tissue and Fluid Bank (GTFB), at the University of Colorado Anschutz Medical Campus, collected urine from women undergoing gynecologic surgery. Urine was obtained from women with ovarian cancer under an IRB approved protocol (COMIRB Protocol 11–0626). Samples were provided as 1 ml aliquots of urine with information on

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patient age, stage and histology of the ovarian cancer. Urine samples were collected during surgery and centrifuged prior to freezing at −80 °C for long term storage. All samples were de-identified and data was analyzed under a second IRB approved protocol at the University of Minnesota (study number: 1610E97724).

2.2. RNA extraction and Quantitative RT-PCR analysis of microRNA

Urine was defrosted once and aliquoted in 100 μl aliquots and refrozen following the addition of 700 μl of Qiazol Reagent (Qiagen, Valencia, CA). Total RNA was isolated from 100 μl of urine using the miRNeasy Mini Kit (Qiagen). The isolation procedure followed the miRNeasy protocol with a few clarifications explained in more detail in Supplemental Table 1. The final elution volume for total RNA from the spin column was the minimum required, 30 μl RNase free water, to allow the maximum concentration of RNA per μl for downstream applications. The concentration and quality of extracted RNA were assessed by spectrophotometry on the NanoDrop 1000 (Thermo Scientific, Waltham, MA). However, the concentration of RNA obtained is much lower than the expected accuracy for the NanoDrop 1000; thus, in the following steps, we ignored concentration estimates and simply used the maximum template volume allowed in the protocol. For example, in the Miscript II kit (Qiagen) for a 20 μl reaction it is possible to reverse transcribe a volume of 12 μls of total RNA for cDNA preparation. Amplifiable RNA extraction and cDNA preparation were confirmed by a positive result for qRT-PCR of a small RNA compared to the cDNA water control. qRT-PCR was conducted using the miScript SYBR Green reagent (Qiagen) with a custom primer for miR-29a-3p 5′-cccTAGCACCATCTGAAATCGGTTA or miR-146a-5p 5′-ggT GAG AAC TGA ATT CCA TGG GTT. We also used the RNU6B primer available from Qiagen for qRT-PCR assays (RNU6B_13). Although in this study, we did not use an internal control for calibration of qRT-PCR results, it is important to mention that an appropriate internal control for exRNA studies is currently controversial. Several helpful studies have examined this controversy and may be of use to those working in this field (27–29).

2.3. RNA precipitation, RNA quality assessment and Illumina Mi-seq methods

Urine is a complex liquid containing EVs, protein, nucleic acids and many other metabolites (30). Tamm-Horsfell is a protein in urine that is known to form networks that trap EVs (31). In the study presented here we extracted RNA from six 100 μl aliquots of urine and then combined these in the following step to reduce the concentration of contaminating substances such as Tamm-Horsfell protein. Total RNA from 600 μl of urine (in 100 μl aliquots) was extracted using the above protocol. The 30 μl aliquots of purified RNA were combined into 1 tube for a total of 180 μls. This was extracted with addition of GenElute LPA as described by the manufacturer (Sigma-Aldrich, St. Louis, MO) as previously described (32). The use of LPA as a carrier is required because alternatives such as glycogen or yeast tRNA are isolated from biological sources and maybe contaminated with small ncRNA (32). After LPA addition, the samples were extracted with low pH phenol (Ambion 9710) and chloroform:isoamyl alcohol (49:1) with a standard ethanol precipitation using 3 M NaAcetate pH 5.2 (protocol included, in Supplemental Table 1).

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2.4. Library preparation and Illumina sequencing analysis

RNA was forwarded to the UCD Genomics and Microarray Core for library construction. In the core facilities, RNA was assessed for quality on the Agilent Bioanalyzer 2100 using the Eukaryote Total RNA Pico Chip (Agilent Technol., Palo Alto, CA). RNA libraries were constructed using a volume of 5 μls of total RNA rather than the recommended concentration of RNA need to prepare the Illumina HiSeq libraries. The TruSeq Small RNA kit uses a 3′ adapter modified to target microRNAs and other small RNAs that have a 3′ hydroxyl group. Enriching for RNA with a 3'hydroxyl allows the detection of RNA that has been enzymatically cleaved by Dicer or other RNA processing enzymes. Small RNA template libraries were sequenced using NGS technology on the Illumina HiSeq2000 platform at the University of Colorado's Genomics and Sequencing Core Facility.

2.5. miR-seq data analysis

The microRNA sequence reads were identified for known and novel microRNA sequences using the program miRDeep. We calculated the expression of the microRNA variants based on normalized read counts and tested for significant differences using ANOVA in R. Galaxy cutadapt was used for each fastq file to remove adapters. Then fastq files were imported into CLC Genomics workbench to identify and count unique small RNAs using two databases as reference (miRBase Release 19 and Homo sapiens GRCh37.57 ncrna). The individual data files of trimmed reads have been uploaded to the publicly available data base at https://doi.org/10.5281/zenodo.801484.

Additional data sets were searched from publicly available data at NCBI on the SRA website (33).

3. Results

In order to identify the repertoire of ncRNAs in urine samples, we developed methods to obtain sufficient RNA for NGS. The amount of total RNA obtained from a one hundred microliter aliquot of urine provided amplifiable RNA for qRT-PCR (Table 1), however, not enough RNA for NGS. In order to collect adequate amounts of RNA for NGS, one milliliter samples of urine were obtained from the GTFB at the University of Colorado and divided into 100 μl aliquots. Each aliquot was then stored at −80 °C with 700 μl of the Phenol reagent, Qiazol for at least one hour (Fig. 1). A final concentration step using low pH phenol was used to combine six samples into one, thus allowing for an increase in total RNA extracted from the same urine sample (Fig. 1).

RNA extracted from eight samples was first assessed for the presence of small RNAs by qRT-PCR for either U6, miR-29a or miR-146a. If the sample were positive by qRT-PCR, the sample was then submitted for library preparation regardless of its RNA integrity number (RIN). Later, the total number of reads were compared across the eight samples and it was found that the total number of reads varied widely (Table 1).

The libraries of ncRNAs in the urine from eight samples consisted of a wide range of RNA molecules including, but not limited to, ribosomal RNAs (rRNA), microRNAs and tRNA fragments (tRFs). The combined NGS results from all eight samples are shown in Fig. 2A,

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which is derived from the total annotated reads of ncRNA and shown as the percent of microRNAs, tRFs and other ncRNAs for all libraries. In Fig. 2B the three categories are shown as percentage of total annotated reads in each individual library.

The overall repertoire of noncoding RNAs varied across samples, with a predominant number of reads falling into the “other ncRNAs” category, although one sample had a higher percentage of tRFs (Fig. 2B, sample 2). The percent of microRNAs varied widely from a few percent to more than 25% (Supplemental Table 2). To better understand the classification of noncoding RNA in the urine samples, we queried the eight samples for the five most highly expressed noncoding RNAs in each library and limited the analysis to those RNAs that were annotated in the GRCH37.57. ncrna data base (Table 2).

The most highly expressed small RNAs were generally fragments related to rRNAs and pseudo rRNAs. Very little is known about the function of extracellular rRNAs and pseudo rRNAs; however, they have been proposed to be processed in response to stress, including oxidative stress (34,35).

Among the noncoding RNAs, microRNAs are currently the most well studied group in urine. Overall analysis of the microRNAs in the eight samples revealed that miR-10b was by far the most highly expressed microRNA in all eight samples followed by miR-10a in five of the eight samples. Other microRNAs that were second to the highest in at least one patient were miR-22 and miR-30a. Interestingly, a microRNA often associated with oncogenesis, miR-21, was only in the top five in one sample (Table 3).

The most highly expressed microRNA in this study, miR-10b, is not well studied in ovarian cancer, although it has recently been implicated in metastasis in other cancer sites (36). Based on a literature review we expected to find miR-146a and miR-29a in urine due to their previous association with ovarian cancer and previous findings of their presence in exRNA samples (23,24,37,38). Neither of these microRNAs were highly expressed in any of the eight samples as detected by NGS.

A large group of reads in the NGS studies derived from tRNAs and belonged to the group of functional noncoding RNAs known as tRFs (39). The small RNA reads in this study were mapped against the known human tRNAs using the GtRNAdb data base (40,41). The most highly expressed tRFs in the eight samples are reported in Table 4.

Highly expressed tRFs in the urine included the tRNA halves as well as a large number of variants differing by only a few bases. One tRF variant of the tRNA halve for tRNA-Glu-CTC (miR-2476) was of particular interest because it had been previously reported as a microRNA in the cow. The miR-2476 had not been previously described in human samples; further, it has been removed from the microRNA data base because of its location near a tRNA, tRF5-Glu-CTC (42). The tRF5-Glu-CTC variant that was previously called miR-2476 is missing a guanine at the sixth base (Table 5A, named tRF5-Glu 1Gv). We examined several NGS studies available at GenBank to determine if this variant of tRF5-Glu-CTC had been observed in other human studies. We queried the publicly available data for the presence of tRF5-Glu 1Gv as well as the most prevalent variant from our urine analysis called tRF5-Glu 2Gv (Table 5B). One study of particular interest, where both

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variants were observed, was a comparison of the RNA cargo in exosomes isolated from the media of ovarian cancer cells, ovarian cancer associated adipocytes, normal adipocytes, cancer associated fibroblasts and normal fibroblasts (33).

The variants of tRF5-Glu (both 1Gv and 2Gv) were found in all 13 samples, with 2Gv being more highly expressed in most samples. The increased expression of the 2Gv is similar to our finding in urine from ovarian cancer patients (Table 5A). Both variants were highly expressed in cancer adipocytes as compared to the other samples. The ovarian carcinoma derived cell line A2780 had increased expression compared to the other three cell lines (43). The top five tRFs from this study were also analyzed for their expression in data bases available at NCBI in the SRA collection (Supplemental Table 3). In this expanded analysis tRF5-Glu was by far the most highly expressed tRF from urinary exosomes. The use of publicly available data to verify the existence of specific variants of tRFs provides a readily available resource to the research community for follow-up studies of NGS analysis. Confirmation of variant expression by their presence in additional publicly available samples may guide the choice for further study of specific exRNAs.

4. Discussion

Liquid biopsies utilizing novel and robust ncRNA targets hold great potential for the development of new biomarkers (4,44). High throughput sequencing studies have fueled the search for novel ncRNA biomarkers from accessible samples including serum, plasma and urine (3,24). The unexpected stability of exRNA and new technologies to deep sequence RNA have led to an explosion of new biomarker studies focusing on ncRNAs in accessible samples (1–4). RNA is a preferred molecule for biomarker discovery in liquid biopsies due to its representation of the physiological state of the organism (44,45). RNA as a potential biomarker in urine was recognized as early as the 1970s when tRNAs were detected at increased levels in urine from cancer patients (46). In these early studies, it was not possible to distinguish the specific noncoding RNA in urine, while it is now feasible with the advent of RNA-seq technologies and the bioinformatics tools to analyze the vast amount of information.

Methods to extract exRNA from accessible samples are widely ranging and varied in the ability to capture amplifiable RNA (11,47). It was initially thought that RNA was only stable in urine if it were protected as cargo in exosomes from bladder and urinary tract cells or from the kidney if there is damage. Thus, studies of exRNA in urine exosomes were initially expected to favor the isolation of ncRNA from the bladder. However, it is now increasingly accepted that exRNA in urine can also come from other tissue sites as well as tumors (48–50).

Many of the recent studies aimed at identifying exRNA in urine through NGS protocols are focused on the ncRNA found in EVs and including the subset known as exosomes (47). The extraction of RNA from urinary extracellular vesicles requires a large volume of urine to ensure that enough RNA will be extracted for downstream applications. The suggested volume of urine for NGS biomarker discovery protocols ranges anywhere from 5 mls to 250 mls of urine (25–27,47). Furthermore, the collection of exosomes requires either

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ultracentrifugation or treatment with a proprietary method such as the Exosome RNA Isolation kit from Norgen (Norgen Biotek Corporation, Thorold, ON, Canada) to concentrate the exosomes (27). Studies of the repertoire of exRNA associated with EVs and specifically exosomal RNA provide a partial understanding of the exRNA present in urine. Methods to extract total RNA from urine are also needed.

Methods designed to conduct NGS on total RNA extracted from urine are less common than exosomal studies. One such study of total RNA extracted from the urine of male goats utilized 500 μl samples successfully to accomplish NGS (51). Other studies based on the extraction of total RNA from a small volume of urine did not attempt NGS, rather these methods were used for microRNA Array studies or qRT-PCR and required as little as 50 μls for analysis (52). The methods we describe in this article require similar volumes of urine and allow the isolation and analysis of total RNA by both qRT-PCR and NGS. Once biomarkers are discovered in pilot studies such as these, it will be possible to scale up to larger studies and develop methods with direct clinical utility.

In order to determine the full spectrum of exRNA in urine, patient samples were requested from the GTFB at the University of Colorado. These samples had been obtained with informed consent and had all been collected during surgery and handled in a consistent manner prior to storage at −80 °C. Consistent collection and storage of samples is required for all studies of exRNA and the importance of collection and storage has been described previously (11,53). Ovarian cancer is a very difficult disease to detect and frequently is found at late stage; thus, one goal of the GTFB is to provide a resource for studies to discover new biomarkers for patient care. In this pilot study, we analyzed the microRNAs and tRFs and grouped the rRNA fragments with all other noncoding RNAs (Fig. 2). The total tRFs, microRNAs and other noncoding RNAs were first analyzed for all samples by percent of total annotated reads. Combining the reads from all samples gives an overview of the types of RNAs in ovarian cancer patient urine (Fig. 2A). However, the grouping of all reads is misleading as individual samples were very different from each other (Fig. 2B). The variability of classes of RNA in patient samples suggests a potential to discover unique biomarkers in urine. Although this proof of concept project provides methods to identify biomarkers in urine, a larger study with the goal of discovering specific biomarkers for ovarian cancer patients will be required.

The top five most highly expressed exRNAs in most samples were dominated by the group of other noncoding RNAs; however, miR-10b, miR-10a and miR-30a were also found in this group (Table 2). The predominant microRNA was by far miR-10b, which was the top microRNA in all samples (Table 3). miR-10a was also one of the top five in all samples and in many samples miR-22 and miR-30a were frequently observed. miR-21, a microRNA reported to be highly expressed in cancer patients, was only observed among the top five molecules in one sample (Table 3). Although the goal of this pilot study was to develop methods to study RNA biomarkers in patient urine, it will be interesting in future studies to determine if there is a role for miR-10a and 10b in ovarian cancer. A literature review revealed that few previous studies of miR-10 in ovarian cancer have been conducted. However, the role of miR-10 has been elucidated in normal granulosa cells of the ovary and is linked to feedback regulation of the TGF-β signaling pathway (54). We also observed the

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presence of miR-10a and 10b in publicly available data of normal fibroblasts and cancer associated fibroblasts, where the miR-10 family was equally expressed in both normal and cancer associated fibroblasts (33). A group of microRNAs expressed in ovarian cancer has been suggested to form a signature predictive of poor outcome in ovarian cancer patients (55); however, the only microRNA included in that signature that is also detected in the top five microRNAs in this study was miR-30d (Table 3). The role of microRNAs in ovarian cancer is still being investigated as is the role of tRFs. These exRNAs are expected to provide new physiological relevant biomarkers for ovarian cancer.

exRNA contains a complex variety of noncoding RNAs, including numerous tRFs and their variants whose complexity is just now being realized (35,56–59). The complexity of tRF expression is in part due to the frequent modification of nucleosides in the mature tRNA, which may interfere with tRF detection in high-throughput sequencing studies (60). In addition to high throughput sequencing, additional methods including qRT-PCR and Northern analysis are routinely used to identify tRFs. However, tRFs are derived from pre- and mature tRNAs making them difficult to distinguish by established assays. Honda et al. have developed methods to specifically analyze tRFs using ligation PCR (61). Future studies aimed at applying tRFs as biomarkers in patient samples will require the development of methods specific to tRFs.

In order to confirm the use of NGS of RNA from urine as a potential method of discovering new biomarkers, we chose one tRF, tRF5-Glu-CTC, for further study. We examined publicly available databases of exRNA isolated from exosomes to determine if tRF5-Glu and its variants are detectable in exRNA from extracellular vesicles (Table 5B). Libraries of exRNA isolated from exosomes obtained from ovarian cancer cell lines, cancer associated adipocytes, normal adipocytes, cancer associated fibroblasts and normal fibroblasts were queried for the presence of tRF5-Glu-CTC (33). The predominant variants from the urine samples were also detected in the RNA from exosomes (Table 5). The use of publicly available data to confirm the expression of previously unidentified exRNAs provides an additional resource to study the numerous exRNAs in urine. The methods described here are readily adapted to other species and other disease conditions for the future development of physiologically relevant urine-based biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this study was provided by the HERA Foundation to MAS and LTB. Funding was also provided by the Whiteside Institute to LTB.

Abbreviations used:

ncRNA	noncoding RNA
exRNA	extracellular RNA

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EVs	extracellular vesicles
tRNA	transfer RNA
tRF	tRNA fragment
rRNA	ribosomal microRNA
qRT-PCR	quantitative RT-PCR
NGS	next generation sequencing
miRNA	microRNA
RNases	ribonucleases

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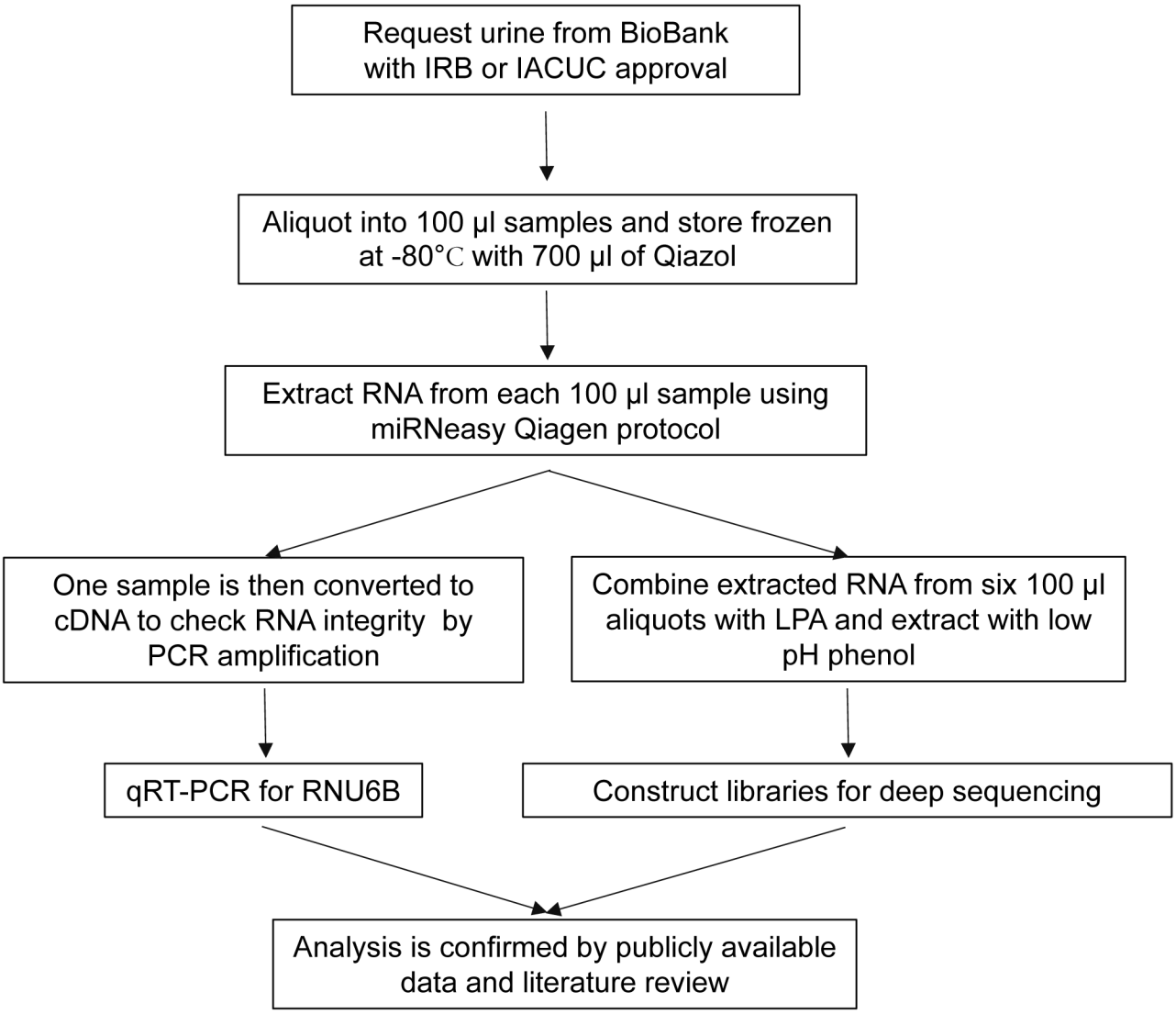

Fig. 1.

Suggested workflow for ncRNA extraction and analysis from urine obtained from patients or animal models. Notes of specific clarification of the method are included in Supplemental Table 1 (a–d).

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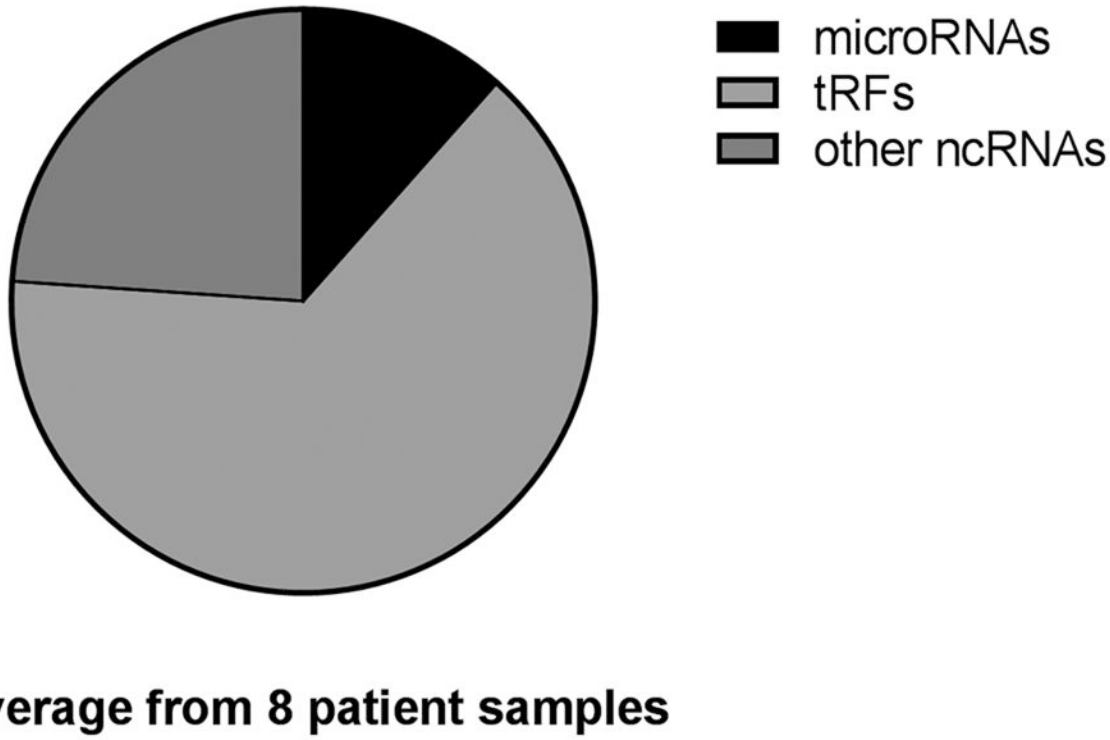

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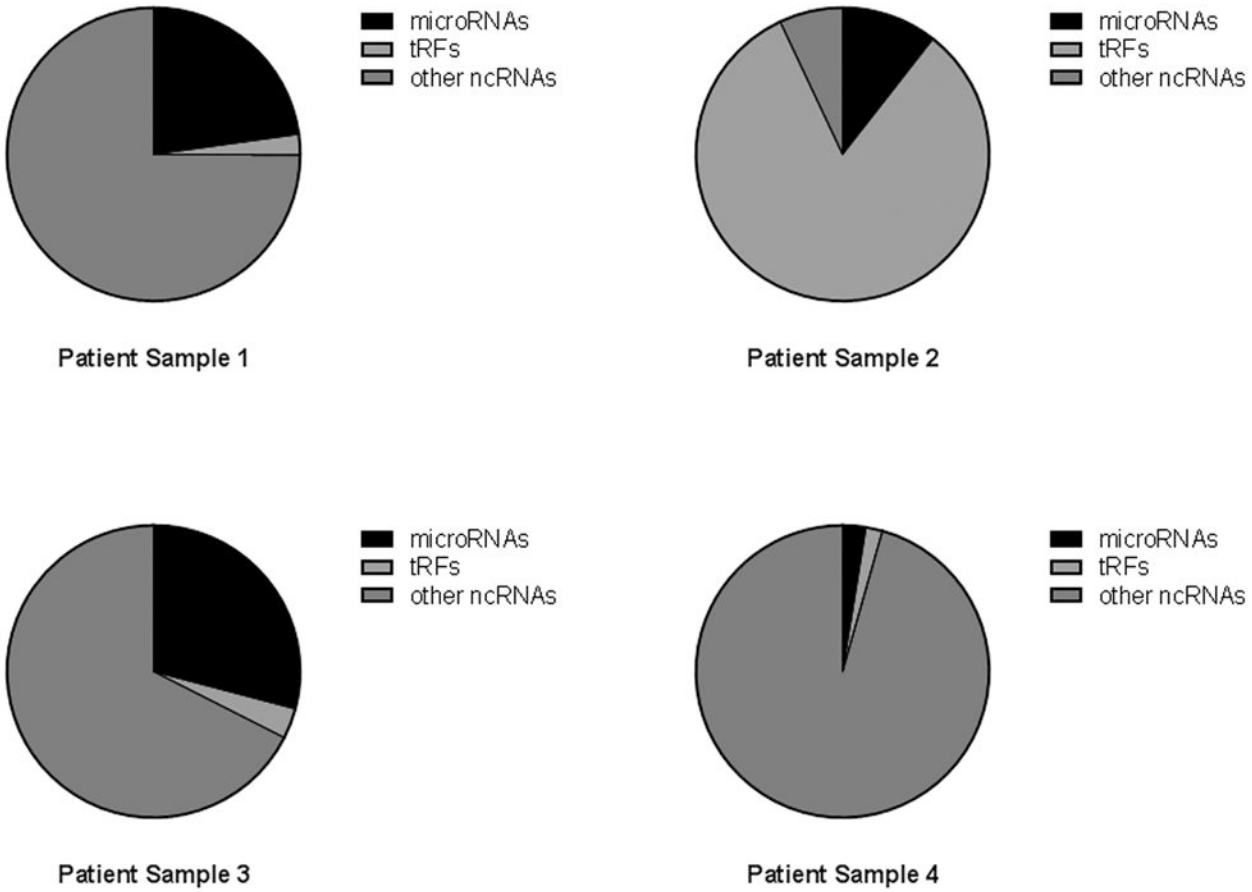

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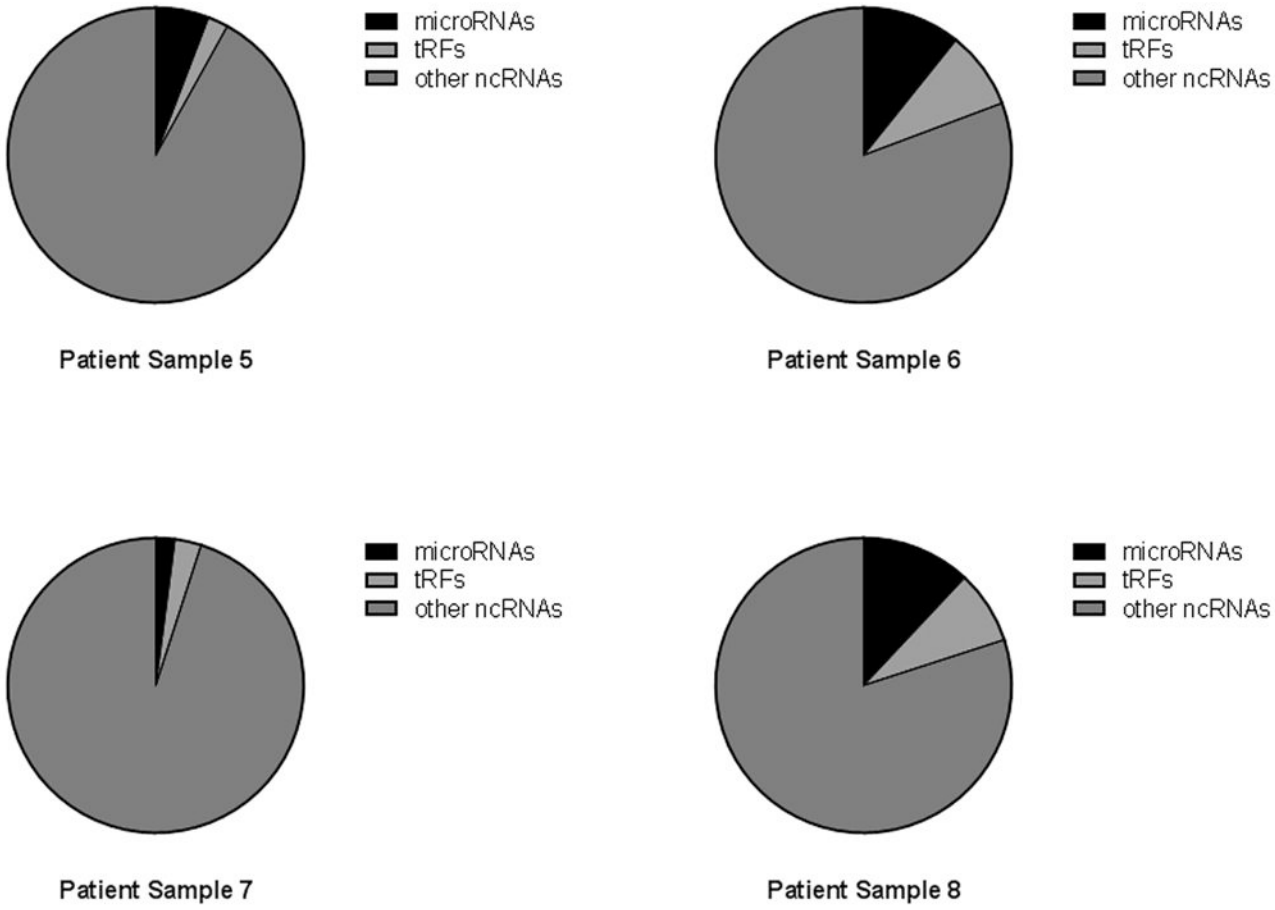

Fig. 2.

Percentage of noncoding RNA. Reads from urine classified as microRNA, tRFs or other ncRNAs. (A) Total reads from 8 urine samples summarized by percent of reads from each sample, microRNAs (black), tRFs (light grey) and other noncoding RNAs (dark grey). (B) The percentage of reads for each individual sample again with microRNAs (black), tRFs (light grey) and other noncoding RNAs (dark grey).

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Table 1.

Total RNA was extracted from eight urine samples and analyzed by qRT-PCR, bioanalysis and RNA-seq. The age, stage and histology for each patient sample is included.

Sample #	Age, stage	Histology	RNA quality (Detectable RNA)	RNA quality (RIN)	Total reads in millions
1	72, IIIC	mucinous adenocarcinoma	U6, miR-29a	2.5	2.6
2	63, IIIC	high grade serous	U6, miR-29a, miR-146a	2.2	49.6
3	48, IIIC	high grade serous	U6, miR-29a, miR-146a	2.6	6.2
4	67, IV	high grade serous	U6	2.5	2.7
5	72, IV	high grade serous	U6	1	1.6
6	57, IIIC	high grade serous	U6, miR-29a, miR-146a	2.5	5.8
7	48, IIC	high grade serous	U6	Less than 1.0	4.8
8	72, IIIC	high grade serous	U6	1.1	2.5

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Table 2.

The top 5 ncRNAs in each of the urine samples as determined by percent of total annotated reads. The Ensembl genome number from GRCH37.57. ncrna is included as well as a sequence name when available.

Patient Samples # \ Top5 ncRNAs	1	2	3	4	5
1	ENST00000466130 (rRNA pseudo gene)	miR-10b	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	ENST00000493956 (rRNA pseudo gene)	ENST00000489202 (rRNA pseudo gene)
2	ENST00000483476 (tRF)	ENST00000486830 (tRF)	miR-10b	miR-30a	miR-10a
3	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	miR-10b	ENST00000489202 (rRNA pseudo gene)	miR-10a	ENST00000493956 (rRNA pseudo gene)
4	ENST00000466130 (rRNA pseudo gene)	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	ENST00000496481 (rRNA pseudo gene)	ENST00000493956 (rRNA pseudo gene)	ENST00000492060 (rRNA pseudo gene)
5	ENST00000493956 (rRNA pseudo gene)	ENST00000479524 (rRNA pseudo gene)	ENST00000474870 (rRNA pseudo gene)	ENST00000474075 (rRNA pseudo gene)	ENST00000463737 (rRNA pseudo gene)
6	ENST00000466130 (rRNA pseudo gene)	ENST00000474885 ENST00000459522 (5.8S ribosomal 1)	ENST00000483476 (tRF)	ENST00000486830 (tRF)	miR-10b
7	ENST00000493956 (rRNA pseudo gene)	ENST00000479524 (rRNA pseudo gene)	ENST00000463737 (rRNA pseudo gene)	ENST00000476674 (rRNA pseudo gene)	ENST00000459949 (rRNA pseudo gene)
8	ENST00000493956 (rRNA pseudo gene)	ENST00000479524 (rRNA pseudo gene)	miR-10b	ENST00000466130 (rRNA pseudo gene)	ENST00000474870 (rRNA pseudo gene)

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Table 3.

The top 5 microRNAs in each of the urine samples as determined by percent of total annotated reads.

Patient Sample # \ Top5 microRNAs	1	2	3	4	5
1	miR-10b	miR-22	miR-10a	miR-205	miR-30a
2	miR-10b	miR-30a	miR-10a	miR-21	miR-22
3	miR-10b	miR-10a	miR-22	miR-205	miR-30d
4	miR-10b	miR-10a	miR-30a	miR-22	miR-205
5	miR-10b	miR-10a	miR-92a-1	miR-92a-2	miR-203a
6	miR-10b	miR-10a	miR-22	miR-205	miR-30d
7	miR-10b	miR-10a	miR-30a	miR-204	miR-4454
8	miR-10b	miR-10a	miR-30a	miR-204	miR-22

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Table 4.

The top 5 tRFs in each of the urine samples as determined by percent of total annotated reads.

Patient Sample # \ Top5 tRF	1	2	3	4	5
1	Glu	Gly	Lys	Val	His
2	Gly	Glu	Val	Lys	His
3	Gly	Glu	Val	Lys	His
4	Glu	Gly	Lys	Val	His
5	Glu	Gly	Lys	Val	His
6	Gly	Glu	Val	Lys	His
7	Gly	Glu	Lys	Val	His
8	Gly	Glu	Lys	Val	His

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Table 5.

The expression of tRF5-Glu-CTC and its variants in urine and human samples. (A) The variants detected by NGS in all eight urine samples. The average in all eight samples is given followed by the range of expression. The length in base pairs refers to the length of the variant. The entire length of tRNA-Glu-CTC-1-1 is included for comparison. (B) The two most prevalent variants of tRF5-Glu-CTC are presented here along with the SRA accession number and type of sample.

Sequence of variants	Average in 8 tumors (range)	Length in base pairs
TCCCTG-TGGTCTAGTGGTTAGGATTCGGC	2926 (14, 22967)	29
TCCCTGGTGGTCTAGTGGTTAGGATTCGGC	4555 (51, 35617)	30
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCG	4044 (17, 31848)	31
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGC	7064 (37, 49667)	32
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCT	7326 (144, 40615)	33
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTC	2079 (70, 12402)	34
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTCT	850 (58, 4303)	35
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTCTC	452 (10, 2463)	36
tRNA-Glu-CTC-1–1		
TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCTCTCACCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA		

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	Accession number	tRF5-Glu 1Gv	tRF5-Glu 2Gv	tRF5-Glu 1Gv percentage	tRF5-Glu 2Gv percentage	Total reads
Ovarian cancer cell line A2780	SRX1550574	186	1439	0.087%	0.670%	214630
Ovarian cancer cell line HeyA8	SRX1550575	43	105	0.015%	0.037%	281330
Ovarian cancer cell line OVCA433	SRX1550576	32	114	0.009%	0.034%	338204
Ovarian cancer cell line SKOV3	SRX1550577	5	25	0.002%	0.010%	256003
Ovarian cancer adipocytes OMT924007	SRX1550585	8065	10261	1.748%	2.224%	461313
Ovarian cancer adipocytes OMT916645	SRX1550586	1901	1380	0.812%	0.589%	234100
Cancer associated fibroblasts CAF866652	SRX1550580	20	1113	0.007%	0.385%	288787
Cancer associated fibroblasts CAF869881	SRX1550581	116	293	0.027%	0.068%	427929
Cancer associated fibroblasts CAF888242	SRX1550582	111	523	0.023%	0.108%	485258
Normal ovarian fibroblasts NOF151	SRX1550578	60	435	0.020%	0.143%	305013
Normal ovarian fibroblasts NOF81000	SRX1550579	76	254	0.023%	0.078%	324908
Normal omental adipocytes OMN050312	SRX1550583	1473	35	0.434%	0.010%	339618
Normal omental adipocytes OMN923075	SRX1550584	84	97	0.031%	0.036%	269810

tRF5-Glu 1Gv, tRF5-Glu 1G variant (CCCTG-TGGTCTAGTGGTTAGGATTCGGC)

tRF5-Glu 2Gv, tRF5-Glu most prevalent 2G variant (CCCTGGTGGTCTAGTGGTTAGGATTCGGCGCT)